DESIGN AND USE OF A UNIQUE SAMPLE CELL
FOR FRONT SURFACE LUMINESCENCE
SPECTROMETRY

By

James Alvah McHard

A Dissertation Presented to the Graduate Council of
the University of Florida
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy

University of Florida
1972

DEDICATION

Without the love, devotion and confidence of my wife, Daisy; my daughter, Sharon; my sons, James and Richard; my mother, Florence McHard; and mother-in-law, Violet Miller, the work presented in this dissertation would not have been possible. To them goes my eternal gratitude and to them I dedicate this manuscript.

ACKNOWLEDGEMENTS

The listing of people to whom I owe thanks for their support is far too long to include them all here. I do want to thank especially Dr. James D. Winefordner for his wise counsel and his never-ending enthusiasm and encouragement. I also want to thank my other counselors, Dr. R. G. Bates, Dr. Luther Arnold, Dr. Gerhard Schmid, and Dr. Carl Stoufer for their patient and sincere assistance.

I express my appreciation also for the cheerful, willing and skillful aid in the design and fabrication of the cell and related components which was accorded by Mr. Arthur Grant and Mr. Daley Burch.

Finally, I want to thank the whole faculty of the Chemistry Department of the University of Florida for giving me this opportunity and their support.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iii

LIST OF TABLES vi

LIST OF FIGURES. vii

Abstract ix

Chapter

I INTRODUCTION. 1

II FRONT SURFACE LUMINESCENCE SPECTROMETRY: ROOM TEMPERATURE 3

Previous Studies. 3

Mathematical Relationships for Fluorimetry and Phosphorimetry 5

Features of the New Design. 8

Fabrication of the New Cell 9

Room Temperature Fluorescence Measurements: Clear Solutions 17

- Instrumentation 17
- Chemicals 17
- Other apparatus 17

Room Temperature Fluorescence Measurements: Colored Solutions 18

Fluorescence at High Concentrations 18

III FRONT SURFACE LUMINESCENCE SPECTROMETRY: PHOSPHORIMETRY. 22

Theoretical Basis for Phosphorescence 22

Sample Cell Modification for Phosphorimetry 23

Measurement of Operating Temperature. 37

Chapter Page

Phosphorescence Data 37

Instrumentation. 37
Chemicals. 40
Procedure. 41

Phosphorescence Analytical Curves. 42

IV QUALITATIVE LUMINESCENCE STUDIES AT LOW TEMPERATURES: FROZEN LIQUIDS 49

Phosphorescence Lifetimes. 49

Character of Spectra at Low Temperatures 54

Environmental Aspects Influencing Luminescence 69

Character of solvent and viscosity 69
Acidity. 78
Multiple peaks 78

Summary Comments on Environment. 79

V QUALITATIVE LUMINESCENCE STUDIES ON SOLIDS 80

VI QUALITATIVE RESULTS OF MULTIPLE INTERNAL REFLECTANCE IR STUDIES. 89

VII PROJECTIONS FOR FUTURE STUDY 96

Design Modifications 96

Luminescence Studies 101

Further Studies on Solids and Viscous Media. 102

VIII SUMMARY. 105

BIBLIOGRAPHY. 107

BIOGRAPHICAL SKETCH 111

LIST OF TABLES

Table		Page
I	Structure and Luminescence Properties of Analyte Molecules Used in Low Temperature Phosphorescence Studies	38
II	Experimentally Determined Phosphorescence Excitation and Emission Peaks (in nm)	46
III	Measured Phosphorescence Lifetimes of Certain Molecules	53
IV	Quenching of Quinine Fluorescence by Heteropoly Acids (room temperature)	84
V	Characteristics of Crystaline Plates for Multiple Internal Reflectance	93

LIST OF FIGURES

Figure Page

1. Schematic diagram of Aminco-Bowman spectrophotofluorometer. 11

2. Geometrical arrangement of FSWC (room temperature design) in standard Aminco cell housing. 13

3. Front view of FSWC (room temperature design) 15

4. Analytical curve for quinine (fluorescence at room temperature). Response in arbitrary units 20

5. Phosphoroscope with windows for intermittently irradiating sample 25

6. Design dimensions of FSWC for low temperature studies. . . 27

7. Vertical arrangement of cooling unit, housing and motor. 30

7a. Insulator design 32

7b. Overhead view of placement of housing components and Dewar flask. 34

7c. Design of phosphoroscope 36

8. Phosphorescence analytical curves for seven molecules. Response in arbitrary units. 44

9. Phosphorescence analytical curves from literature. 48

10. Calculated plot of lifetimes vs. temperature 52

11. Phosphorescence emission spectra (-140°C) of tryptophan (25 ppm aqueous mixture 10^{-3} M in H_2SO_4). Exciting wavelength 290 nm. 56

12. Fluorescence emission spectra (-140°C) of tryptophan (25 ppm aqueous mixtures, 10^{-3} M in H_2SO_4). Exciting wavelength, 290 nm. 58

Figure Page

13. Phosphorescence emission spectra (-140°C) of quinine (25 ppm aqueous mixtures, 10^{-3} M in H_2SO_4). Exciting wavelength, 340 nm. 60

14. Phosphorescence excitation and emission spectra of quinine in acidic solution compared to basic solution (-140°C). Concentration 25 ppm. Solvent-propylene glycol (25 percent in H_2O). 62

15. Fluorescence spectra of quinine (25 ppm). Comparison of aqueous propylene glycol and aqueous methanol solutions (all in 10^{-3} M in H_2SO_4) at -140°C. Meter sensitivity 10^{-7} 64

16. Phosphorescence spectra of p-nitrophenol (25 ppm) in basic and acidic solution (-140°C).. 66

17. Room temperature fluorescence spectra of quinine in H_2O, 0.05 M in H_2SO_4. 68

18. Effect of solvent on emission of a hypothetical molecule 71

19. Fluorescence spectra of quinine at different exciting wavelengths 240-340 nm; 380 nm; and 420 nm. Aqueous 0.05 M H_2SO_4. Quinine concentrated 2 ppm. 75

20. Emission characteristics of 6 ppm of quinine in dioxane-water mixtures. 77

21. Cubooctahedral configuration of heteropoly anions of silicon and phosphorus 82

22. Phosphorescence spectrum of quinine adduct with tungsto-silicic acid. 88

23. Schematic diagram showing light path through multiple internal reflectance plate 91

24. Comparison of infrared spectra. 95

25. Schematic arrangement of cell and cooling system with temperature control device. 99

Abstract of Dissertation Presented to the
Graduate Council of the University of Florida in Partial
Fulfillment of the Requirements for the Degree of Doctor of Philosophy

DESIGN AND USE OF A UNIQUE SAMPLE CELL FOR FRONT SURFACE LUMINESCENCE SPECTROMETRY

By

James Alvah McHard

August, 1972

Chairman: James Dudley Winefordner
Major Department: Chemistry

Front surface illumination and retrieval has provided the most widely accepted method to date for obtaining luminescence spectral data from viscous, turbid, and highly colored liquids and from pastes and solids. Prior designs of sample cells for front surface luminescence have been limited in their utility because they require specially constructed cells which are often fragile and difficult to clean, need cumbersome optical path arrangements, and have been restricted to fluorescence studies at room temperature, and/or require specially adapted environmental conditions. The sample cell described herein overcomes many of the disadvantages of previous designs. It is durable, easy to clean, and versatile. The versatility feature includes; ability to accept a wide variety of sample types and adaptability to both fluorescence and phosphorescence. The design of the cell is described and examples are given of its broad range of usefulness with liquids, both viscous and highly colored, and also with solids.

Analytical curves are presented for seven analyte molecules; atropine, caffeine, p-nitrophenol, phenylalanine, quinine, tryptophan, and yohimbine. Results are given on the fluorescence characteristics of heteropoly acids (HPA's) of phosphorus and silicon as their adducts with drugs. It is shown by the data presented that both fluorescence and phosphorescence energies and apparent quantum efficiencies are markedly affected by the environment of the molecule. A study is also reviewed of the possible qualitative usefulness of multiple internal reflectance in the infrared for the study of the HPA drug adducts.

CHAPTER I

INTRODUCTION

This dissertation deals primarily with the development and use of a sample cell allowing front surface illumination for fluorimetry and phosphorimetry. The notable features of the design are its simplicity, adaptability, and versatility.

Front surface fluorescence analysis is not a new concept (1, 2, 3). Its use is necessary if one is to study solids, highly colored, or turbid substances, or concentrated solutions of analyte. For example, in measuring fluorescence from chromatographic paper strips (4, 5, 6), a number of approaches have been described for admitting exciting light to the strip surface and measuring the emitted radiation at some displaced angle from the same side.

Elaborate arrangements have been devised (7, 8, 9, 10) to study solids as well as highly absorbing or turbid liquids. In nearly all situations, especially for liquids, the sample cell serves as a container as well as a means of allowing exposure of the analyte to suitable irradiation. This often means that the excitation and emission beams each pass through a layer of glass or quartz, with the attendant reduction of intensities. As a further complication, spurious emission bands from the glass or quartz (2, 11) may adulterate the analyzing beam.

Since most of the more common sample holders require glass or quartz, additional disadvantages are inherent. Meticulous attention must be accorded to keeping the surfaces free from scratches, finger marks, and dirt. Fragility is a problem and can be a disadvantage of the cells, especially those made from quartz. Solids and pastes which adhere to the glass surface amplify the cleaning problem where scrubbing of difficultly accessible areas is involved.

Luminescence spectrometry, taken comprehensively to mean fluorimetry and phosphorimetry, has become by now an established method of analysis especially for biological and environmental analysis problems (12, 13, 14, 15). The study presented here offers an important new way to measure more difficult types of samples.

CHAPTER II

FRONT SURFACE LUMINESCENCE SPECTROMETRY: ROOM TEMPERATURE

Previous Studies

A more detailed description than that given in the Introduction of some of the unique approaches to the problem of frontal surface analysis will be helpful in understanding the intricacies of cell design.

In an attempt to adapt the principle of attenuated total reflection (ATR) to fluorescence, Hirschfeld (7, 16, 17) has designed a brass sample carriage to mount the ATR components so that they fit into a Beckman DU Spectrophotometer. The optical train in the sample compartment consists of a vertical mirror inclined at 30° to the spectrophotometer axis. The beam is thus reflected to a lateral mirror and from there to a silica microscope slide wherein it is multiply internally reflected. The microscope slide is positioned by the carriage so that the front face rests against the entrance to the analyzing monochromator. The opposite face of the slide is fitted with glass pieces cemented into place to form a sample compartment. The inside surfaces of these cemented pieces are silvered to avoid loss of light. The cell has been used in a study of the intensity/concentration ratio of fluorescein solutions up to 1000 ppm. Comparisons have been made of solutions with and without added solids (suspensions of calcium carbonate). Linearity in the response curve has been reported up to several hundred ppm. In a later study, Hirschfeld (18) has treated the geometrical aspects of the design and has adapted Fahrenfort's mathematical treatment of ATR (19) to the new device.

An entirely different concept, but one resembling more closely the sample positioning arrangement described in this manuscript, has been proposed by Ainsworth (10, 20). In operation, light from the excitation monochromator impinges on the front surface of a glass or quartz cell mounted at an angle in the cell housing. The mounting is such that a 5° cone of the fluorescent beam is selected for the analyzing monochromator.

An arrangement for front surface observation of fluorescence has been made available commercially by the American Instrument Company (Silver Springs, Maryland) for the Aminco-Bowman Spectrophotofluorometer (3). This accessory accepts the usual square quartz cuvette. In this arrangement, the cell is situated at the rear of the housing. The emitted light is taken at a slightly displaced angle from the normal and then by a system of small mirrors directed to the analyzing monochromator.

The above designs, in general, have been useful for liquids and turbid or colored solutions, but the handling of solids and pastes is difficult if not impossible.

Another more recent and quite unique concept for frontal analysis has been described by Guilbault (21, 22, 23). In this design the sample cell consists of a silicone rubber pad prepared as needed by pressing the raw gum between glassine paper and allowing to cure. The author's description of the exact preparation of the cell strips is not entirely clear. From the account in his publication it appears that some sort of V-shape in the pad is required. After curing, the rubber must be cut into strips of the right shape. This undoubtedly requires some skill to obtain the correct size and form. The strips are apparently discarded after each use, making it necessary to cut and prepare a new pad for each analysis. The finally cured and sectioned strip is placed on a metal cell holder fabricated to fit into the cell compartment of the standard Aminco-Bowman spectrophotofluorometer. Because the sample has to be mounted horizontally to avoid loss of it by gravity, the instrument is turned on edge. The exciting beam of radiation is directed onto the sample in a direction parallel to the surface of the cell holder.

None of the designs described above has been adapted to low temperature fluorimetry or phosphorimetry.

Mathematical Relationships for Fluorimetry and Phosphorimetry

As an aid to understanding some of the analytical parameters involved in luminescence analysis a short resumé of pertinent equations is given. A number of excellent reviews (1, 2, 24, 25, 26, 27) present detailed treatments of the theory. For the purposes of this presentation, the equations of Winefordner, St. John, and McCarthy are well suited.

It is to be noted that certain assumptions have been made in developing the general equation (Eq. 1), which are not necessarily all valid for the situations described in this dissertation. However, variations in individual parameters are kept to a minimum by experimentally adopting a uniform cell placement and keeping controllable parameters the same throughout any series of related measurements.

It is also to be noted that the general expressions for phosphorescence and fluorescence are similar in appearance and differ only in the magnitudes of some of the terms. The general equation and the definition of terms follows:

$$E_s = \left(\frac{\gamma \alpha R_L G k^2 R_d^2 W^4 \phi_s I_o}{4\pi A_s \Delta\lambda}\right) [1 - \exp(2.3 a_s b C_s)] \qquad \text{Eq. 1}$$

E_s = signal magnitude at the detector output

γ = photoanodic sensitivity of the phototube

α = observation efficiency due to rotating shutter (phosphorimetry)

R_L = magnitude of phototube resistor

k = monochromator factor (includes monochromator solid angle, transmission factor, and slit area)

R_d = reciprocal linear dispersion of monochromator

ϕ_s = overall quantum efficiency (power emitted per power absorbed)

I_o = intensity of the exciting source

A_s = surface area of emitting sample

$\Delta\lambda$ = half intensity width wavelength interval over which emission occurs

a_s = molar absorptivity coefficient of the sample

b = cell depth

C_s = concentration of analyte species

The assumptions used in deriving this general equation are: 1) the excitation and emission monochromators have exactly the same optical parameters; 2) the entire sample can be excited; 3) the luminescence can be observed from the entire sample; 4) the sample absorbs only weakly at the emission wavelength.

When the sample concentration is low the exponential term (i.e., the term in brackets $[1 - \exp(2.3a_sbC_s)]$) can be assumed to be equivalent to $2.3a_sbC_s$ (by ignoring all terms in the McLaurin series expansion except the first). In this case, the equation becomes:

$$E_s = \frac{2.3\,\gamma\alpha R_L G a_s b C_s k^2 R_d^2 W^4 \phi_s I_o}{4\pi A_s \Delta\lambda} \qquad \text{Eq. 2}$$

For the alternate situation in concentrated solutions, the exponential term given above approaches the value of one, and:

$$E_s = \frac{\gamma\alpha R_L G k^2 R_d^2 W^4 \phi_s I_o}{4\pi A_s \Delta\lambda} \qquad \text{Eq. 3}$$

The signal in concentrated solutions as represented by Eq. 3 is no longer proportional to the concentration of analyte but depends entirely on the quantum efficiency of the sample (assuming no source fluctuation).

These equations can be applied to the sample cell described in this manuscript as long as the angle of placement with regard to the incoming and outgoing beams remains constant. Actually, the tangent of the angle of displacement from 90° should be included, as shown by Ainsworth (10), but, if this is kept the same, the equation can be corrected by the inclusion of another constant.

Features of the New Design

In contrast to the lack of versatility and sometimes complicated optical arrangements of the previous frontal sample cell designs, the one which will be described here has maximum utility in being able to accommodate a wide variety of samples. Furthermore, the cell has been developed so that phosphorimetry as well as fluorimetry can be carried out at room temperature as well as at low temperatures. The new design has a number of unique features. There is no window in the sense that there is no cell material (e.g., glass) interposed between the exciting beam and emitting beam. In this dissertation, the cell will be therefore often referred to as FSWC (front surface windowless cell) to avoid repetitious wording in some of the discussions. Other outstanding features of the design are: 1) the ease of introducing the sample; 2) ease of cleaning; 3) durability (i.e., it is not fragile like glass and can be dropped without damage); and 4) versatility.

The versatility attribute needs further amplification. FSWC is versatile in the wide variety of samples which can be handled, i.e., solids, colored liquids, viscous liquids, and pastes. It is also versatile in the ease of adaptation from fluorescence to phosphorescence. Although variation in materials of construction has not been a part of this investigation, there is no reason why a wide variety of starting materials could not be adapted including plastics, metals, and even glass if so desired.

Fabrication of the New Cell

The schematic diagram of the Aminco-Bowman spectrophotometer is shown in Figure 1. The housing for the standard sample cell is shown in heavy lines and is marked as H. The standard 1 cm^2 X 10 cm long liquid cell fits snugly in the area outlined in dashes. Appropriate slits and apertures are placed in the entry and exit ports.

The concept of FSWC as constructed for the initial room temperature studies is featured by a special geometrical configuration. This configuration allows the cell to be placed in the standard mount without any modifications of the housing. The cell design and its geometry are seen in Figure 2. The sample cavity is a circular well cut into the face of the brass plate at the position shown in Figure 3. This centers the opening in the screen (which actually serves as a window) on the incoming exciting beam of radiation and also allows the fluorescence to be observed from the same excitation area. The edges of the plate have been bevelled so that the angle of incidence is displaced by 5° toward the normal (i.e., 40° instead of 45°). This displacement moves the peak of the specular reflectance beam to the right of the exit slit. In the early work on the cell design, it was felt that a slightly convex surface was most desirable and if the filling volume is adjusted to make the surface slightly convex then the displacement of the reflectance will be even greater. Later work, however, seems to indicate that a slightly concave liquid surface gives better reproducibility. In this latter case, a better displacement of the specular reflectance could possibly be attained by placing the cell with respect to the incoming light in the opposite

Figure 1.--Schematic diagram of Aminco-Bowman spectrophotofluorometer.

L	xenon lamp
M	excitation monochromator
M'	emission monochromator
H	housing assemply for sample cell
P	photomultiplier housing

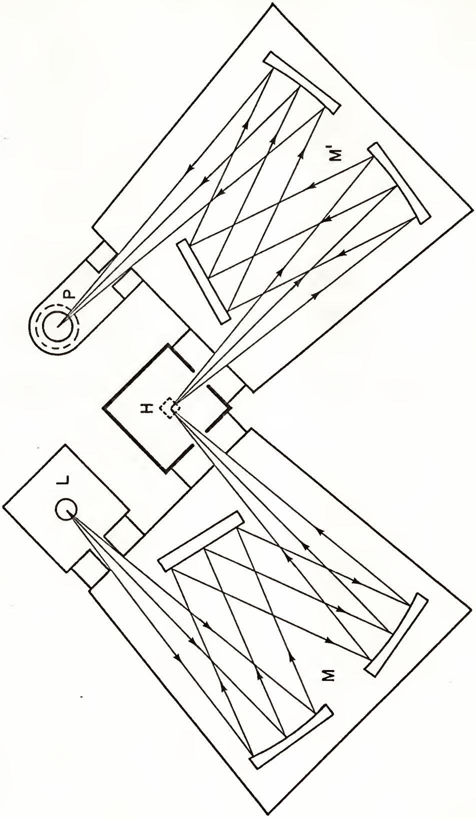

P
M'
H
L
M

Figure 2.--Geometrical arrangement of FSWC (room temperature design) in standard Aminco cell housing.

C	sample cavity (cross section)
S	screen covering

Inset at upper left showing front view of screen

W	opening in screen to allow sample irradiation

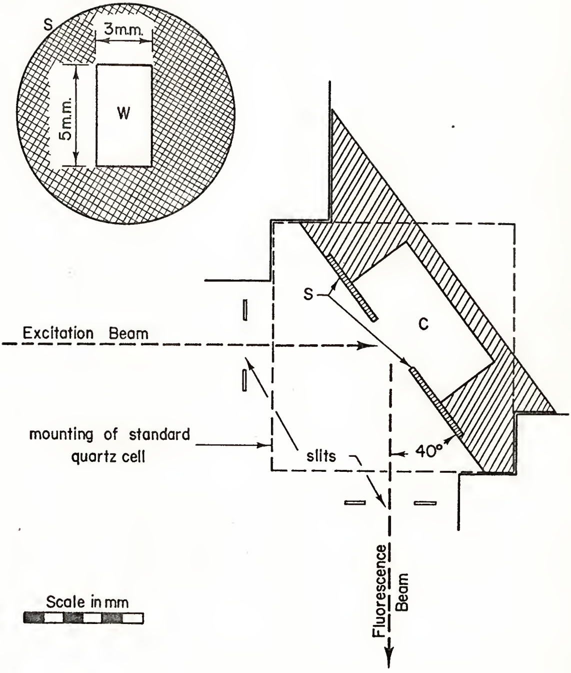
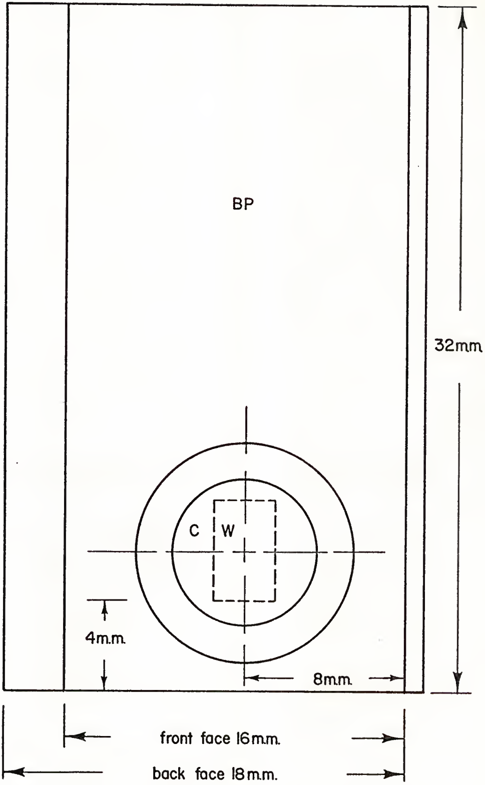

S
3m.m.
5m.m.
W
S
C
Excitation Beam
mounting of standard quartz cell
slits
40°
Fluorescence Beam
Scale in mm

Figure 3.--Front view of FSWC (room temperature design).

BP	brass plate (4.75 mm deep)
C	cell cavity
W	opening in screen


BP
C
W
32mm
4m.m.
8m.m.
front face 16m.m.
back face 18m.m.

direction (i.e., 50° instead of 40°). Investigation of the optimum angle of displacement could be a subject for future investigation. In the design to be described in a later section dealing with the phosphorimetry experiments, the cell can easily be modified so that it may be rotated to any desired angle.

During the development stages, some of the early cell models had screens both front and back of the sample cavity. This feature reduced spectral reflection from the back surface which tended to act as a light mirror. However, these designs had a larger cross sectional area and some types of samples were difficult to retain in the cavity. To overcome a tendency for the sample to seep out, the cavity has been reduced in size to an area of 50 mm^2 (8 mm diameter), and the depth of the cell increased to 3.75 mm to minimize the mirror effect. The back surface has been left completely closed, and a screen of monel (50 mesh) containing a 5 mm x 3 mm opening press-fitted into a counter-bore over the sample cavity. The volume of sample required is approximately 150 μl depending on the exact dimensions of the cavity. Liquids remain in place by surface tension. No particular difficulty in retention using the final configuration has been experienced unless liquids of low surface tension like some alcohols, hydrocarbons, or certain other organic liquids especially those of low viscosity are used as solvents. To further reduce the tendency to migrate and to minimize surface aberrations, a mixture of glycerol (25% by volume) and water has been used for most of the room temperature fluorescence measurements made with this design. The increase in viscosity of the medium provided by the glycerol enhances retention in the cavity and decreases surface turbulence.

Room Temperature Fluorescence Measurements: Clear Solutions

For the study made to demonstrate the utility of the cell for room temperature fluorescence, quinine sulfate was selected for a typical analyte because it had previously been a widely-used reference material (12, 28, 29).

Instrumentation.--An Aminco-Bowman spectrophotofluorometer with a 150-watt xenon arc lamp (see Figure 1) and a potted 1P28 photomultiplier tube (American Instrument Company, Silver Springs, Maryland) was used for the fluorescence studies.

Chemicals.--The quinine sulfate was obtained from Nutritional Biochemicals Corporation and used without purification. The glycerol was Baker's Analysed. The water was deionized (not further purified) and the sulfuric acid was Baker and Adamson electronic grade.

Other apparatus.--All glassware was soaked in concentrated sulfuric acid, allowed to stand over night in 20 percent sulfuric acid and finally rinsed several times before use with deionized water.

The analytical curve obtained for quinine solutions of varying concentrations is shown in Figure 4 (curve A). Individual points represent the average of three readings taken at each concentration. It is evident that linearity is preserved over several orders of magnitude.

The reproducibility (percent relative standard deviation) of the readings was about 10 - 15 percent in most cases. There was annoying variability in the background signal which accounted for the somewhat disappointing reproducibility. Part of this was due to source variation, part to some evident photomultiplier tube instability, and part to variations in the spectral reflectance because of sample surface turbulence. The latter has been referred to above and was more evident at some times than others for some obscure reason. The use of a polarizer and a filter in the excitation beam moderated this problem, but only one polarizer was available; optimization of these remedies could probably be obtained by the use of an additional polarizer in the exit beam along with an exit beam filter (12, 28).

Room Temperature Fluorescence Measurements: Colored Solutions

Since one of the more unique applications of the FSWC is its use with turbid or highly colored solutions, an investigation of this type was carried out. A colored mixture of Superchrome Red B (Allied Chemical) and indigo carmine was prepared. The final mixture which contained ~ 1000 ppm of each dye turned out to resemble the color, character, and density of blood. Also, fortunately, the mixture was of itself non-fluorescent and served as a suitable matrix for observation of fluorescent analytes in a color dense medium. The analytical curve is linear and, as is portrayed by curve B in Figure 4, the applicability to analysis at high concentrations is implied.

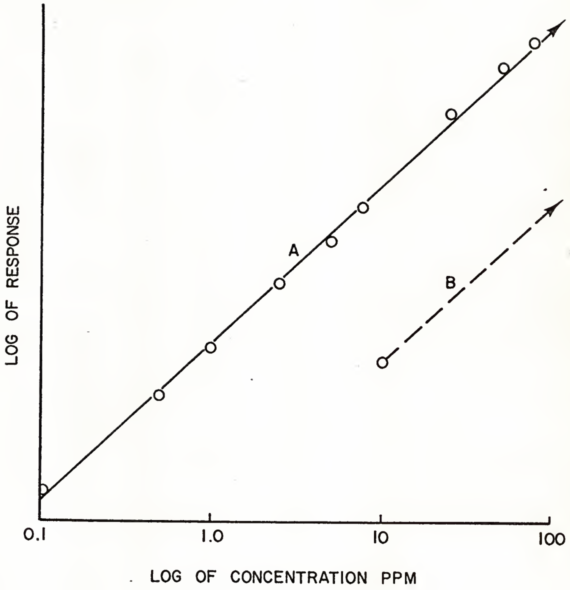
Fluorescence at High Concentrations

A general phenomenon usually observed with concentration-response curves in luminescence spectrometry is the leveling off at high concentrations; that is, the curve flattens out and is no longer concentration-dependent but follows the character predicted by Eq. 3. The reason for this behavior is that the absorption factor in brackets

Figure 4.--Analytical curve for quinine (fluorescence at room temperature). Response in arbitrary units.

A graph of points obtained from 25 percent aqueous glycerol (0.05 M in H_2SO_4)

B graph of points obtained from colored matrix (superchrome red B and indigo carmine; aqueous 0.05 M H_2SO_4)


LOG OF RESPONSE
A
B
0.1
1.0
10
100
LOG OF CONCENTRATION PPM

(in addition, the pre- and post-filter effects should be considered which also lead to a flattening of the analytical curve) become increasingly important with increasing concentration of analyte. Front surface cell designs minimize the pre- and post-filter problem. However, with the highly-colored dye solutions, no flattening of the curve was observed (especially when the incident light intensity was reduced). It would not be possible to reduce light intensities when studying fluorescence in the conventional manner with the square quartz cell because at high concentrations there would be too much absorption of exciting light, and light would not reach far enough into the cell to excite analyte molecules in the area of observation. In the highly-colored medium described, true front surface fluorescence can be approached.

This latter feature introduces the possibility of using highly-colored matricies as a general medium. Further research, as will be summarized later in the discussion, could be carried out using deeply colored non-fluorescent dyes. It is also possible that fluorescence could be measured in a dark matrix, e.g. suspensions of carbon black in a viscous medium like mineral oil.

CHAPTER III

FRONT SURFACE LUMINESCENCE SPECTROMETRY: PHOSPHORIMETRY

Because the cell FSWC proved to be useful for room temperature fluorescence spectrometry, it became of interest to develop a modified cell for use in low temperature fluorimetry and phosphorimetry. Therefore, modifications were made in the design to allow for these studies.

Theoretical Basis for Phosphorescence

Phosphorimetry differs from fluorimetry in the manner of monitoring the emitted light from the sample. The actual production of the phosphorescent molecule also must allow for changes to take place in the excited states following the initial absorption of radiant energy. These changes are more extensive and time-consuming than the changes which take place during the fluorescent process. The many excellent reviews (1, 2, 12, 24, 25, 26, 27, 31) of the theory involved describe and explain these events, and so the theory will only be briefly reviewed here. For phosphorescence to be observed, time must be allowed for the molecule after excitation to undergo electronic spin transformation to the triplet state. To permit intersystem crossing and subsequent radiational transition to the ground state, the character of the environment of the molecule must be such that collisional and molecular migrational processes which dissipate the energy in nonradiational pathways are minimized. The usual way to achieve these conditions is to immobilize the matrix by reducing the temperature, although it is possible to observe phosphorescence of some molecules in certain glasses at room temperature.

The most common way to discriminate between phosphorescence and fluorescence is by time resolution, i.e., by use of a phosphoroscope (chopper). One of the more common forms of phosphoroscope is portrayed diagrammatically in Figure 5. This was the design used in the present study. The device has two openings in a rotating can which, in operation, surrounds the sample cell. With this simple rotating can, the phosphorescence radiation of long lifetime phosphors can be separated from the luminescence of short lifetime luminescent molecules. Generally speaking, fluorescence lifetimes are several orders of magnitude smaller than the phosphorescence lifetimes. Fluorescence lifetimes are usually less than 10^{-6} second and phosphorescence lifetimes are usually greater than 10^{-3} second.

Sample Cell Modification for Phosphorimetry

The front surface windowless cell (FSWC) needed some changes in geometry to allow for installation of the phosphoroscope and a Dewar flask to hold the coolant. Provisionally, then, the cell was constructed from copper rod, the details being shown in Figure 6 (piece labelled C). Copper was selected because of its superior heat transfer characteristics. The cell base was machined with positioning pins to rest snugly on a copper cold finger (piece labelled F in Figure 6), the bulk of which is submerged in liquid nitrogen during operation.

Figure 5.--Phosphoroscope with windows for intermittently irradiating sample.

P	phosphoroscope
W, W'	phosphoroscope windows

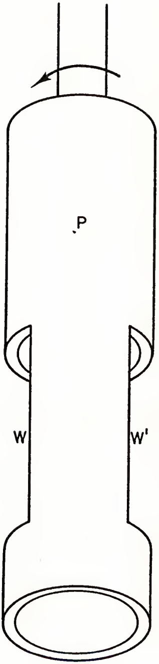
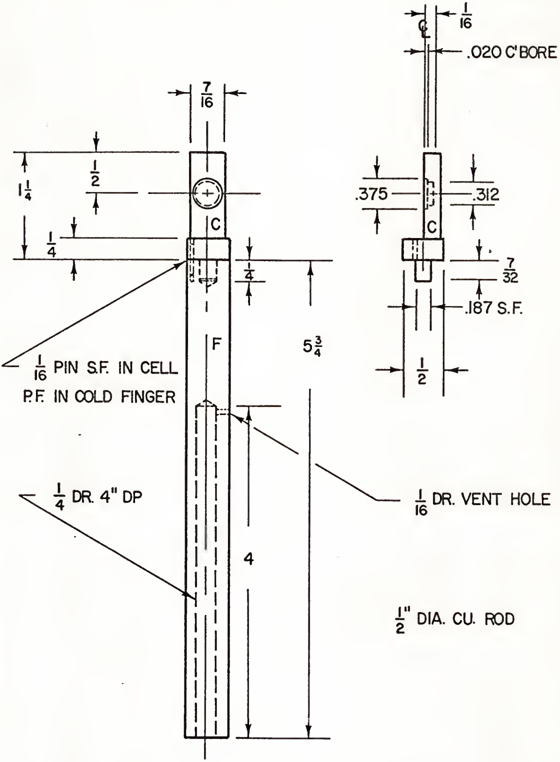

P
W
W'

Figure 6.--Design dimensions of FSWC for low temperature studies.

C sample cell

F cold finger (showing also seat arrangement with cell C in place)



$\frac{1}{16}$
.020 C'BORE
$\frac{7}{16}$
$1\frac{1}{4}$
$\frac{1}{2}$
$\frac{1}{4}$
C
.375
.312
C
$\frac{1}{4}$
$\frac{7}{32}$
.187 S.F.
F
$5\frac{3}{4}$
$\frac{1}{16}$ PIN S.F. IN CELL
P.F. IN COLD FINGER
$\frac{1}{2}$
$\frac{1}{4}$ DR. 4" DP
$\frac{1}{16}$ DR. VENT HOLE
4
$\frac{1}{2}$" DIA. CU. ROD

In the final assembly the seat for the cell at the top of the cold finger extends about 1 - 2 inches above the surface of the N_2 and is anchored to the housing by means of a Teflon mount. The mount provides insulation from the housing which in design was enlarged from that shown as area H in Figure 1, and machined to fit snugly against both monochromators. The Teflon mount (shown in Figure 7a) has 1/4 inch holes to allow for escape of cold nitrogen vapors around the cell. Also, it has a locking pin to position the cold finger radially in the housing. The nitrogen reservoir consists of a glass Dewar flask with a capacity of approximately 600 ml. The assembly without the cell and hood described below is shown in Figure 7. Additional details are given in related drawings labelled 7a, 7b, and 7c.

Initial tests of the functioning of the cooling system and cell mount revealed that under steady state conditions (achieved after a few minutes cooling), the rate of escape of cool nitrogen vapor escaping through the cell housing was insufficient to prevent condensation of moisture. To correct this, an aluminum hood was installed over the Teflon to confine the escaping vapors to an area within a 1/16 inch opening just surrounding the cell, and additionally a provision was made for a stream of dry nitrogen from a compressed gas storage tank to be bubbled at about 1 cc/sec through the liquid nitrogen supply. This forces an adequate volume of vapor to surround the cell and drive moisture out of the housing.

The cell cavity was designed to be exactly the same diameter as that described previously for the fluorescence work but shallower.

Figure 7.--Vertical arrangement of cooling unit, housing and motor.

M	motor for phosphoroscope
P	phosphoroscope
T	entrance and exit light tunnels
F	cold finger (without cell in place)
D	Dewar reservoir for liquid nitrogen (capacity ~600 ml).
I	insulator (teflon) mount for cold finger

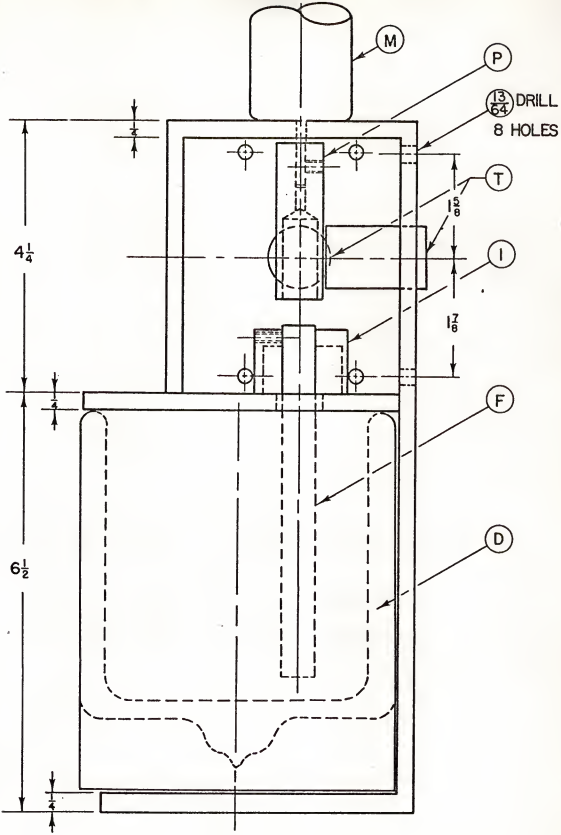
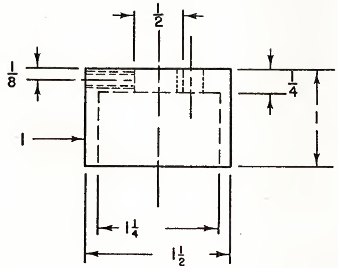

M
P
$\frac{13}{64}$ DRILL
8 HOLES
T
I
F
D
$4\frac{1}{4}$
$6\frac{1}{2}$
$\frac{1}{4}$
$\frac{1}{4}$
$\frac{1}{4}$
$1\frac{5}{8}$
$1\frac{7}{8}$

Figure 7a.--Insulator design

Vertical cross section

Overhead cross section
showing placement and
size of openings.


1/2
1/8
1/4
1
1
1 1/4
1 1/2

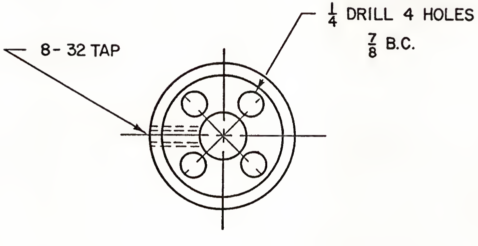

8-32 TAP
1/4 DRILL 4 HOLES
7/8 B.C.

Figure 7b.--Overhead view of placement of housing components and Dewar flask.

T, T'	entrance and exit light tunnels
D	dotted outline of top of Dewar flask

Large square--housing outline for Dewar flask

Small square--housing outline for sample cell (front face of cell is positioned at the intersection of the light beams)

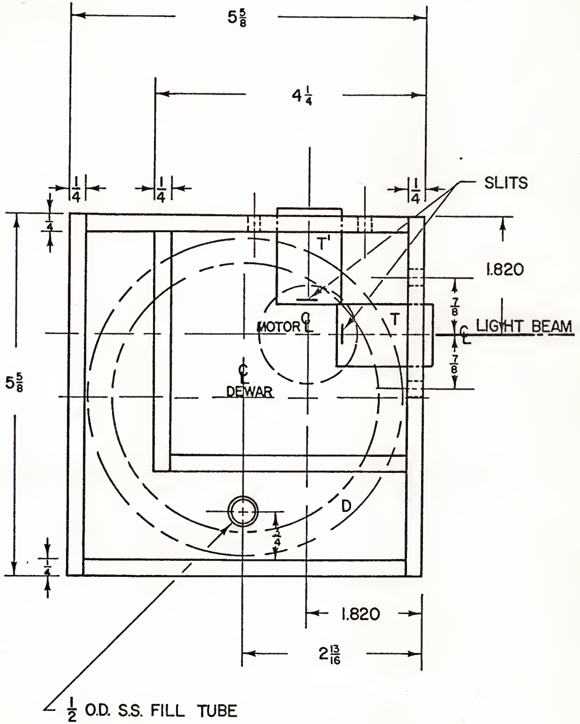
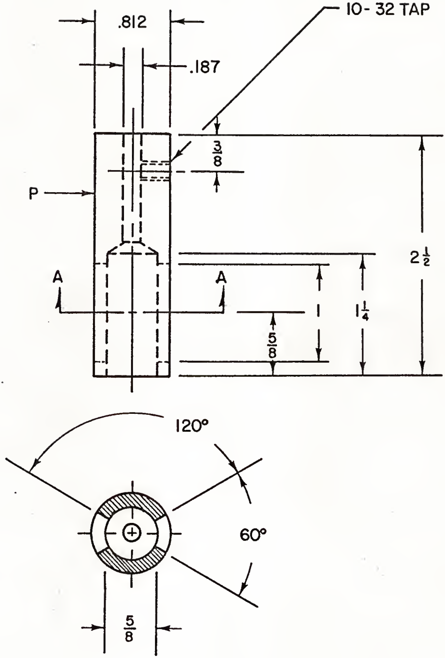

5 5/8
4 1/4
1/4
1/4
1/4
1/4
SLITS
1.820
T'
T
7/8
LIGHT BEAM
7/8
MOTOR ℄
℄ DEWAR
5 5/8
D
3/4
1/4
1.820
2 13/16
1/2 O.D. S.S. FILL TUBE

Figure 7c.--Design of phosphoroscope.

vertical dimensions

horizontal cross section
showing phosphoroscope
windows (angular apertures)


10- 32 TAP
.812
.187
3/8
P
2 1/2
A
A
1 1/4
5/8
120°
60°
5/8

That is, the depth of the cavity of the new design is approximately 2 mm rather than 3.75 mm. This alteration in depth has reduced the sample volume requirement from approximately 150 µl to approximately 80 µl.

Measurement of Operating Temperature

The temperature of the liquid in the cell under operating conditions was measured by installing a copper-constantan thermocouple through a hole in a blank cover for the housing. The temperature sensitive end was immersed and anchored by wire in a mixture of solvents (the composition of which will be described later) contained in the cell. The thermocouple was calibrated prior to use with room temperature water, dry ice-acetone and liquid nitrogen. Two separate readings taken from a graph of the calibration gave temperatures of -140°C ± 2 degrees. Although this measured temperature is nearly 60° above the boiling point of liquid nitrogen, as will be discussed later, it is possible in actual operation to obtain good phosphorescence signals.

Phosphorescence Data

To demonstrate the utility for phosphorescence, analytical curves were obtained on the analyte molecules shown in Table I. The values given in the table for the wavelengths of excitation and emission are quoted from the literature (12).

Instrumentation.--The combination of monochromator units used for this low temperature study was the Aminco-Bowman arrangement described earlier (see Figure 1).

TABLE I

STRUCTURE AND LUMINESCENCE PROPERTIES OF ANALYTE MOLECULES USED IN LOW TEMPERATURE PHOSPHORESCENCE STUDIES

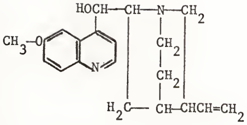
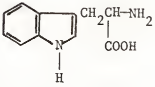
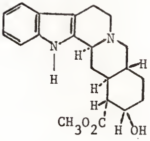
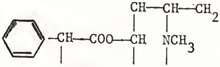
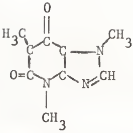
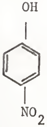
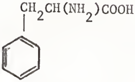
	Fluorescence		Phosphorescence	
	Exλ	Emλ	Exλ	Emλ
 quinine[a]	350	450	340	500
 tryptophan[a]	287	348	295	440
 yohimbine[a]	270	360	290	410
 atropine[b]	Non-fluorescent		w*	410

TABLE I (continued)

	Fluorescence		Phosphorescence	
	Exλ	Emλ	Exλ	Emλ
 caffeine[c]	Non-fluorescent		285	440
 p-nitrophenol[d]	-	-	355	520
 phenylalanine [c]	Non-fluorescent		270	385

*continuum excitation

[a] source: Nutritional Biochemicals, Corp., Cleveland, Ohio

[b] source: Merck & Co., Inc., Rahway, New Jersey

[c] source: Eastman Chemicals, Kingsport, Tennessee

[d] source: Chemical Service, Media, Pennsylvania

The source lamp was a 150-watt xenon short-arc illuminator distributed by Eimac (Division of Varian). The lamp and lamp housing are physically separated from the excitational monochromator in contrast to the integrated housing unit incorporated into the standard Aminco-Bowman unit.

Provision was made for the installation of machined slits at the entrance face of the excitation monochromator.

Guides for replaceable standard slits were provided for at the cell end of the entrance and exit light tunnels (area T, Figure 7) in the housing.

Chemicals.--The analyte specimens were obtained from the sources listed and were used without further purification.

The matrix selected for these studies was a solution of propylene glycol (Fisher Scientific, laboratory grade) 25 percent by volume in deionized water. The total mixture including analyte was 0.05 M in sulfuric acid in all instances.

The rationale for selecting the propylene glycol water mixture was that room temperature fluorescence studies, made prior to construction of the phosphorescence assembly, had shown propylene glycol to have a low background approaching that of deionized water. Furthermore, the resulting frozen matrix appeared to be a finely divided, glistening-snow at the temperature of operation. This latter attribute has been only recently recognized as being desirable for low temperature studies (11, 32).

Procedure.--The sample volume, at first, was measured carefully with a calibrated micro syringe. As mentioned earlier in the discussion, the volume required was ~ 80 µl, (actually 85 - 87 µl); this amount of liquid completely wetted the inside of the sample cage, i.e., that volume element within the cavity and below the screen. This optimum volume, on freezing, expanded slightly to form a completely level surface at the face of the opening in the screen. More recent experience indicated that it was more expedient and just as reproducible to use throw-away glass micro pipettes and judge the volume visually against a lighted background.

The sample cell containing the matrix and analyte was placed in position on the cold finger and the phosphoroscope cover adjusted in place on top of the housing. Time was allowed for the cell and sample to reach a steady state temperature. The optimum time for this period was about 3 minutes before each reading. This was the time period required for the signal to reach a maximum and level off.

Three replicate readings were taken at each concentration. For each replicate, the cell was removed, flushed with water, then three times with isopropanol and dried in a stream of air. This flushing and drying procedure required about 30 seconds.

For some of the analytes, especially in the case of tryptophan, there was a tendency for the signal to decrease after a long exposure. For tryptophan, this time period was about four and one-half minutes.

After three readings were taken at each concentration, a task consuming about 15 minutes, it was necessary to re-level the liquid nitrogen again to the top of the reservoir. For the purpose of judging the height of the liquid, an unsilvered strip had been provided on the Dewar flask. Filling was accomplished by pouring the liquid nitrogen through a copper funnel inserted at each filling into a port hole in the cover of the Dewar (Figure 7b). This port hole was plugged after filling with a teflon stopper to prevent escape of the vapors via that route instead of through the housing.

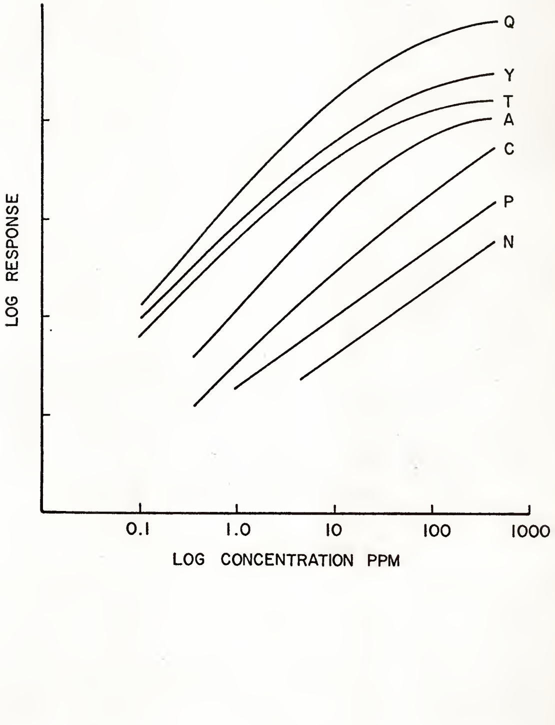
With the modifications described in the section on design, moisture condensation on the sample cell was not a problem as long as the residence time of the cell in the housing was less than 15 - 20 minutes. Some condensed water appeared around the cell seat at the top of the cold finger but this in no way interfered with the operation. In all instances, the cell was easily removed; that is, it did not freeze to the cold finger. Removal was achieved by grasping the top of the cell with a pair of pliers, to avoid direct contact with the fingers, and gently lifting straight up.

Phosphorescence Analytical Curves

The results obtained for the analytical curves are shown in Figure 8. The low concentration ends of the curves terminate at values estimated to be the minimum detectable concentrations using the definition of Winefordner, St. John, and McCarthy. "The limiting detectable sample concentrations is that concentration resulting in a value E_s (signal) equal to the mean background signal obtained under the same experimental conditions used for the sample" (ref. 27, p. 97). As will be discussed in more detail later, the environment of a molecule markedly influences the phosphorescence signal, e.g., the pH of the

Figure 8.--Phosphorescence analytical curves for seven molecules. Response in arbitrary units.

Q	quinine
Y	yohimbine
A	atropine
T	tryptophan
C	caffeine
P	phenylalanine
N	p-nitrophenol


Q
Y
T
A
C
P
N
LOG RESPONSE
0.1
1.0
10
100
1000
LOG CONCENTRATION PPM

solution is important (p-nitrophenol and tryptophan give much better signals in basic media).

The experimentally determined excitation and emission peaks are given in Table II.

The analytical curves generally follow the trends shown for similar molecules in the published literature. For comparison, analytical curves obtained for three of these same analytes, caffeine, quinine, and atropine, by Winefordner and Tin (61) are given in Figure 9. As observed in these earlier studies, there is a tendency for the response of the analytes to level off at high concentrations as predicted by equation 3. In the investigation reported here, however, this leveling off is seen to be not as severe as that found in earlier studies (note the literature curve for caffeine).

Apparently, even with the front surface cell, self-absorption can be a factor at high concentrations. An interesting possibility, which was not investigated because of the inconvenience of changing the slit at the source in the current design, would be to reduce the intensity of the excitation beam as pointed out earlier in the discussion on room temperature fluorescence. This might allow for more linearity in the response at higher concentrations.

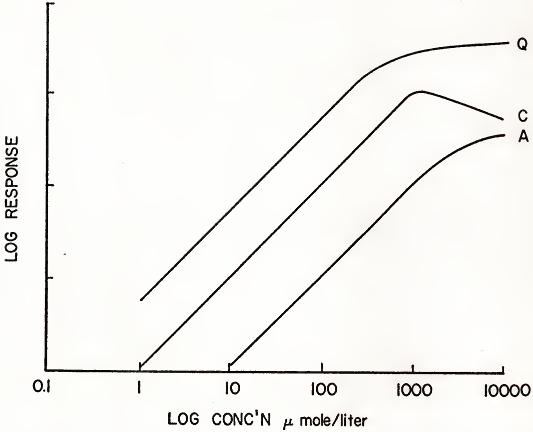
TABLE II

EXPERIMENTALLY DETERMINED PHOSPHORESCENCE EXCITATION AND EMISSION PEAKS (IN NM)

Analyte	Excitation Peak	Emission Peak
atropine	225	375
caffeine	285	440
p-nitrophenol	360	500
phenylalanine	265	360
quinine	355	500
tryptophan	290	430
yohimbine	290	430

Figure 9.--Phosphorescence analytical curves from literature (Winefordner and Tin, Ref. 61).

q	quinine
c	caffeine
a	atropine


LOG RESPONSE
Q
C
A
0.1
1
10
100
1000
10000
LOG CONC'N μ mole/liter

CHAPTER IV

QUALITATIVE LUMINESCENCE STUDIES AT LOW TEMPERATURES: FROZEN LIQUIDS

The studies reported in the literature (2, 33, 34, 35, 62) on the fluorescence of molecules at low temperatures are not nearly as extensive as those of molecules at room temperature, probably because of the lack of suitable commercial equipment. When fluorescence studies are performed with the Aminco spectrophotofluorometer with phosphoroscope attachment but without the phosphoroscope itself (36), i.e., with the quartz sample cell in liquid nitrogen, the scattered light from the bubbles in the liquid nitrogen and from the highly curved cylindrical quartz surface surrounding the sample is high. Consequently, it is impossible in a practical manner to discriminate the sample signal from the scattered background. It was of interest, therefore, to test the adaptibility of the present FSWC cell system to low temperature fluorescence and to qualitatively observe the appearance of the emission spectra both of fluorescence and of phosphorescence.

Phosphorescence Lifetimes

With regard to temperature, it was surprising to find that, even though the operating temperature was well above that used in the usual studies, the phosphorescence signal, as determined at the detection limit, approached the values reported in the literature, certainly in most cases within an order of magnitude. Winefordner, et al. (27) have calculated the effect of temperature on the phosphorescence signal of a hypothetical molecule as shown in Figure 10. From Figure 10, it would be predicted that the signal at 135°K might be considerably smaller than that at 77°K because the quantum yield which influences the signal is a function of lifetimes and the lifetimes are seen to decrease exponentially with increasing temperature.

The effect of temperature on the phosphorescence signal is apparently not directly related to the experimental lifetimes obtained in this study. Lifetimes of four of the analyte molecules were measured by interrupting the light entering the excitation monochromators with a guillotine shutter (27). The time for the phosphorescence signal to decay to a value equal to 1/e of the previous value was measured on a time base recorder, and this time was taken as the phosphorescence lifetime. These results are given in Table III. The lifetimes for quinine and yohimbine are near literature values but somewhat lower. The values for nitrophenol and tryptophan are higher than the reported values. In the case of nitrophenol, the somewhat higher result may be rationalized because one-half second is about the lower limit of determinability by this procedure, and any measurement error would be magnified. For tryptophan, however, there is no obvious rationale for the unusually long lifetime. One possible explanation may lie in the peak selected for measurement of the phosphorescence signal. As will be observed later on, tryptophan exhibits three phosphorescence peaks. The peak for which the lifetime is reported in the literature is for the most energetic (highest frequency), while the peak selected

Figure 10.--Calculated plot of lifetimes vs. temperature (ref. 27).

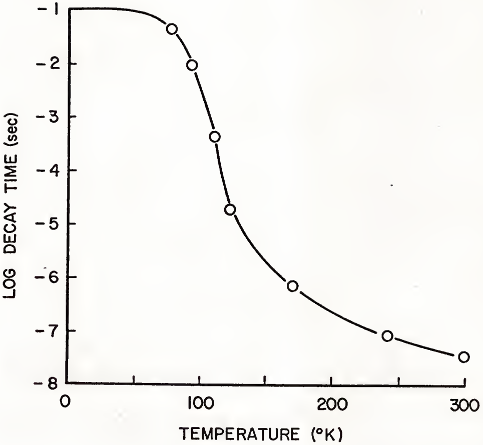

-1
-2
-3
-4
-5
-6
-7
-8
0
100
200
300
LOG DECAY TIME (sec)
TEMPERATURE (°K)

TABLE III

MEASURED PHOSPHORESCENCE LIFETIMES OF CERTAIN MOLECULES

	Experimental Lifetimes (in sec.)	Literature Value Lifetimes (in sec.)
p-nitrophenol	.55	<0.2
quinine	.95	1.3
tryptophan	7.5	1.5
yohimbine	7.0	7.4

for this study is the broad middle energy one of the three. It may be that these peaks have different lifetimes. The time base recorder which was used developed a fault in the high speed gear, necessary for the lifetime measurement, and the lifetimes were not repeated. This aspect could be worthy of some future study, although for the purposes of this dissertation, it is not a crucial point since the intent is to report interesting observations of a general nature to demonstrate primarily the wide analytical applicability of the new cell design.

Character of Spectra at Low Temperatures

The qualitative studies are exemplified by the spectra given in Figures 11, 12, 13, 14, 15, 16, 17, and 22. All, except the room temperature quinine spectrum, were obtained at -140°C. Because an XY recorder was not available, a time base recorder was used, and the wavelengths were recorded by pencil jabs at the proper point in time. This procedure, although somewhat inconvenient, worked surprisingly well and was reproducible to within 5 nm which is as good as the accuracy of the calibration of the wavelength drums on the monochromators.

The concentration of analyte in all instances for these qualitative studies was 25 ppm because this gave signals well above any background effects. The principal aspects intended to be investigated were: (1) effect of solvent composition; (2) effect of pH; and (3) effect of temperature.

Figure 11.--Phosphorescence emission spectra (-140°C) of tryptophan (25 ppm aqueous mixture 10^{-3} M in H_2SO_4). Exciting wavelength 290 nm.

PG	propylene glycol (25 percent in H_2O)
M	methanol (10 percent in H_2O)
B	blank solutions (25 percent propylene glycol)

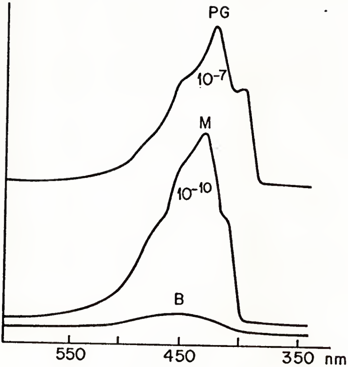

PG
10^{-7}
M
10^{-10}
B
550
450
350 nm

Figure 12.--Fluorescence emission spectra (-140°C) of tryptophan (25 ppm aqueous mixtures, 10^{-3} M in H_2SO_4). Exciting wavelength, 290 nm.

PG	propylene glycol (25 percent in H_2O)
M	methanol (10 percent in H_2O)
B	blank (25 percent propylene glycol)

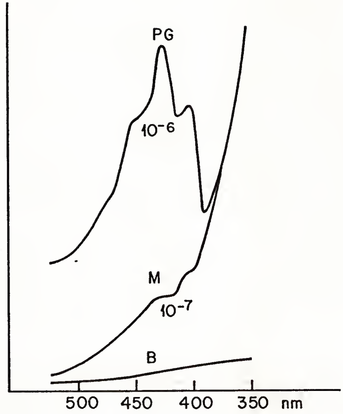

PG
10⁻⁶
M
10⁻⁷
B
500
450
400
350
nm

Figure 13.--Phosphorescence emission spectra (-140°C) of quinine (25 ppm aqueous mixtures, 10^{-3} M in H_2SO_4). Exciting wavelength, 340 nm.

PG	propylene glycol (25 percent in H_2O)
M	methanol (10 percent in H_2O)
B	blank (25 percent propylene glycol)

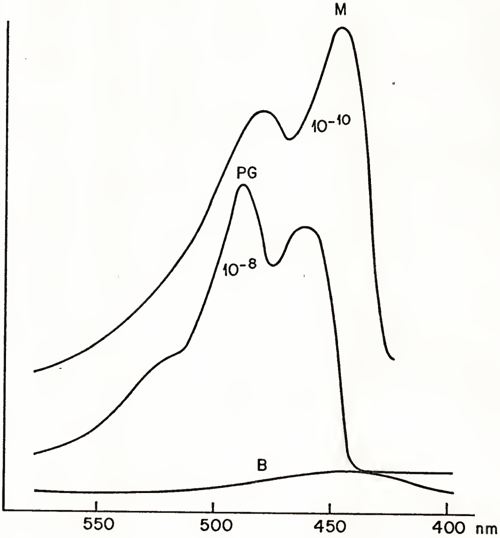

M
10-10
PG
10-8
B
550
500
450
400 nm

Figure 14.--Phosphorescence excitation and emission spectra of quinine in acidic solution compared to basic solution (-140°C). Concentration 25 ppm. Solvent-propylene glycol (25 percent in H_2O).

Basic Solution pH approximately 10

Acidic Solution approximately 10^{-3} M H_2SO_4

I emission spectrum, basic solution

II excitation spectrum, basic solution

III emission spectrum, acidic solution

IV excitation spectrum, acidic solution

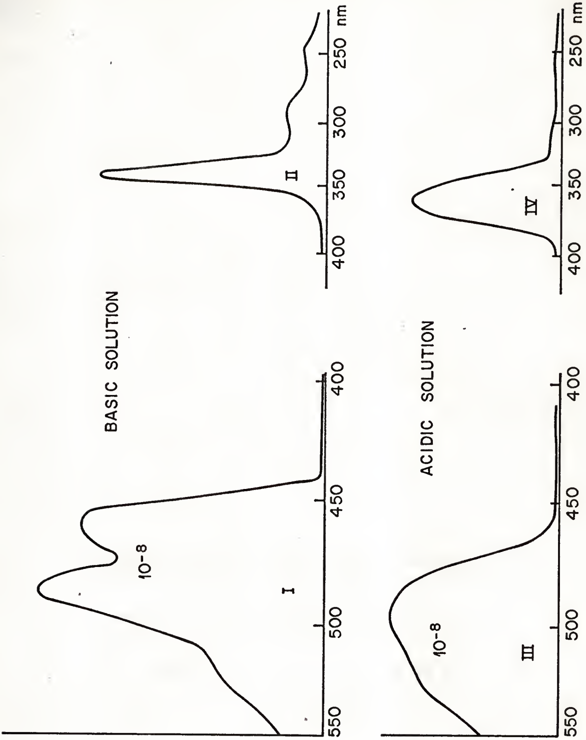

BASIC SOLUTION
ACIDIC SOLUTION
I
II
III
IV
10-8
10-8
550
500
450
400
400
350
300
250 nm
550
500
450
400
400
350
300
250 nm

Figure 15.--Fluorescence spectra of quinine (25 ppm). Comparison of aqueous propylene glycol and aqueous methanol solutions (all in 10^{-3} M in H_2SO_4) at -140°C. Meter sensitivity 10^{-7}.

QPG	quinine in propylene glycol (25 percent)
QM	quinine in methanol (10 percent)
PGB	propylene blank (25 percent)
MB	methanol blank (10 percent)

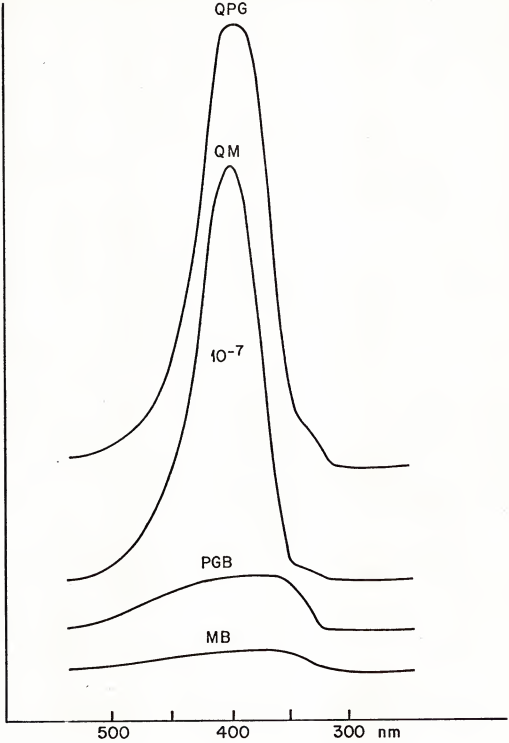

QPG
QM
10⁻⁷
PGB
MB
500
400
300 nm

Figure 16.--Phosphorescence spectra of p-nitrophenol (25 ppm) in basic and acidic solution (-140°C).

PGb	propylene glycol (25 percent in H_2O) pH approximately 10
PGa	propylene glycol (25 percent in H_2O) approximately 10^{-3} M in H_2SO_4
B	blank (25 percent propylene glycol, 10^{-3} M in H_2SO_4)

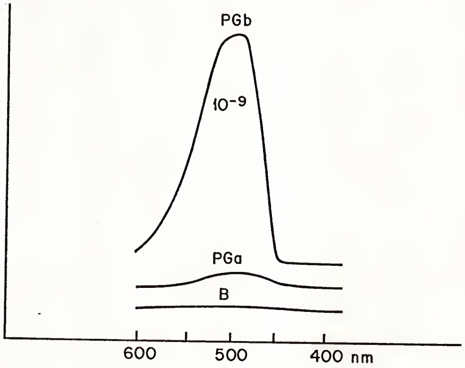
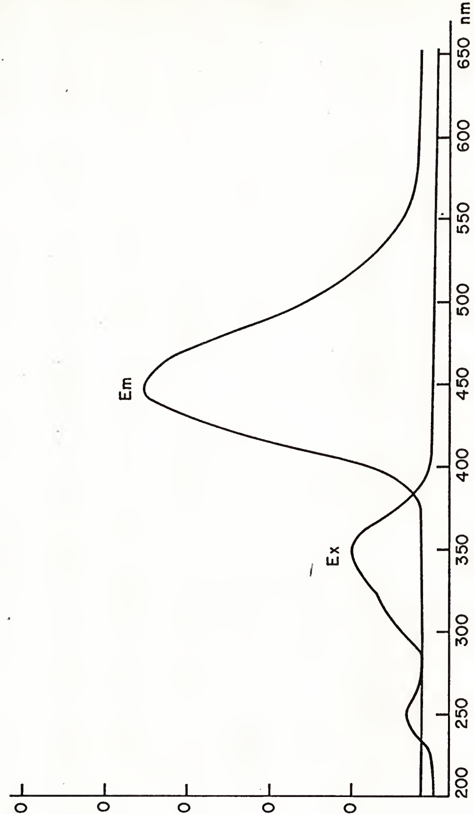

PGb
10⁻⁹
PGa
B
600
500
400 nm

Figure 17.--Room temperature fluorescence spectra of quinine in H_2O, 0.05 M in H_2SO_4.

Ex excitation spectrum-emission wavelength set at 450 nm

Em emission spectrum-excitation wavelength set at 250 nm


50
40
30
20
10
200
250
300
350
400
450
500
550
600
650
nm
Em
Ex

Environmental Aspects Influencing Luminescence

There has been considerable attention given, especially in the more recent literature (2, 25, 26, 35, 38) with regard to the nature of the matrix which surrounds the molecule and the influence it has on luminescent behavior. The polarity of the solvent, hydrogen bonding, and viscosity, as well as solvent temperature, are all regarded as having significant effects.

Character of Solvent and Viscosity.--Both Parker (2) and Wehry (38) explain the solvent interaction energy relationships of excitation and emission (for fluorescence) by diagrams like that shown in Figure 18. The process of both absorption and emission result in a molecule which has dipole energies in the final state, different from that of the starting state. Then, if the environment of the molecule is flexible enough, relaxations take place to adjust to the new dipole configuration. These solvent energy adjustments are, of course, superimposed on the other energy changes taking place. These other changes are, for the most part, transformations from high energy excited singlet levels to lower energy excited singlets and attendant vibrational cascades. All these processes are believed to precede, in most instances, the radiational transition to the ground state, and the net result contributes to the observation that fluorescence occurs at lower energies than does absorption.

If the environment can not relax rapidly, then the energy changes shown in Figure 18 may not take place, and the subsequent radiational transitions are displaced to correspondingly higher energies. Such is the case in viscous or frozen materials.

Figure 18.--Effect of solvent on emission of a hypothetical molecule.

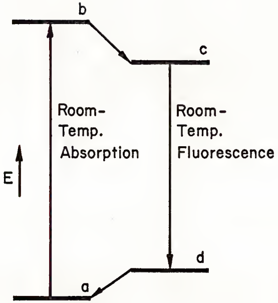
From Parker (ref. 2) and Wehry (ref. 38).

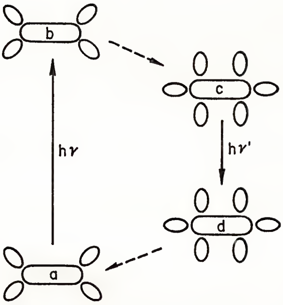
a→b absorption energy from ground state equilibrium with solvent to excited non-equilibrium state with solvent

b→c relaxation of excited state configuration to new solvent equilibrium

c→d emission from equilibrium excited state with solvent to non-equilibrium ground state

d→a relaxation of ground state configuration to starting equilibrium state


b
c
Room-
Temp.
Absorption
Room-
Temp.
Fluorescence
E
a
d


b
c
hγ
hγ'
d
a

Babu and Mugiya (35) use this reasoning to interpret the blue shift sometimes observed in low temperature fluorescence. Their specific explanation is that decreasing the solvent temperature increases the rigidity of the solvent molecules and solvent relaxation to the new dipole configuration in the excited state, an event which precedes fluorescence at room temperature, is interfered with.

It has recently been observed by Sharafy and Muszkat (60) that the normally non-fluorescent cis-stilbene and sterically-hindered trans-stilbenes become strongly fluorescent in very viscous media. The fluorescence of these compounds according to the authors is a steep function of viscosity, due to a solvent free volume (and viscosity) dependent rate constant for the internal conversion ($S_1 \rightarrow S_o$) process.

Viscosity is considered by Parker (2) to be the main parameter determining the rate of diffusion-controlled reactions. Because most quenching processes result from the interaction of the excited analyte molecule with other solvent and impurity molecules in their immediate vicinity, and because both fluorescence and phosphorescence quantum yields depend on the concentration of excited analyte species, viscosity is a prime factor.

A noteworthy qualitative observation made in the present study was an unusually large shift in the position of the fluorescence peak for quinine at -140°C compared with that at room temperature (Figures 15 and 17). Quinine has been a commonly accepted standard for fluorescence studies (3, 12, 29). At room temperature in aqueous acid solutions, the emission peak for quinine appears invariably at

450 nm whether the excitation is done at 250 nm or 350 nm, the two evident absorption maxima (Figure 17). It has been shown by Chen (39), however, that at higher exciting wavelengths the emission band also moves to higher wavelengths. These experiments were repeated and the observations confirmed by Fletcher (40) and also the latter reported that in neutral dioxane water mixtures the emission peaks shifted dramatically to wavelengths less than 400 nm (Figures 19 and 20).

The observation of the shift in the energy of the fluorescence of quinine at low temperatures may be a result of the change in rigidity of the matrix and therefore similar to the findings of Babu and Mugiya and of Sharafy and Muszkat. The observations of Chen and Fletcher made on the shifts at room temperature in different solvents have not been explained. The shift in wavelength of the fluorescence with exciting wavelengths (noted also by Chen and Fletcher) is a point of current controversy as commented on below.

Somewhat related solvent effects were noted in the present study to affect phosphorescence intensities and energies. It was, perhaps, somewhat fortuitous that the choice of solvent for the quantitative phosphorimetric studies reported here was 25 percent propylene glycol in water. As pointed out earlier, at -140°C, the frozen matrix resulting from this mixture was a hard glistening snow. In contrast, the other mixture which was compared in the qualitative studies was 10 percent methanol in water. The latter, instead of being solidly frozen, was more of a mush. The phosphorescence signals obtained from the methanol water mixture, as can be seen from Figures 11 and 13 for tryptophan and quinine, were much weaker than those using propylene

Figure 19.--Fluorescence spectra of quinine at different exciting wavelengths 240-340 nm; 380 nm; and 420 nm. Aqueous 0.05 M H_2SO_4. Quinine concentration 2 ppm. (From Fletcher ref. 40).

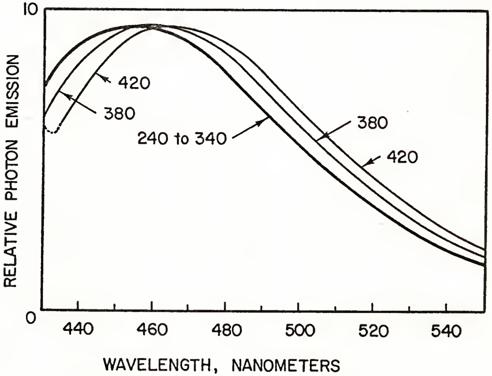
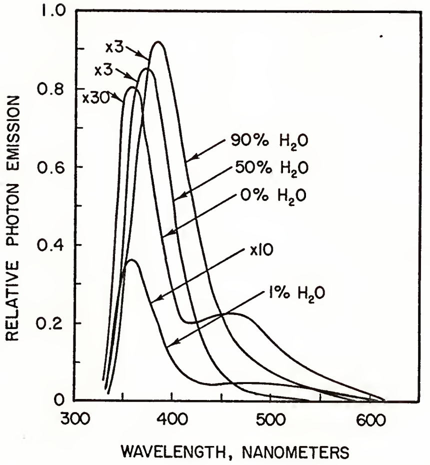

10
0
RELATIVE PHOTON EMISSION
420
380
240 to 340
380
420
440
460
480
500
520
540
WAVELENGTH, NANOMETERS

Figure 20.--Emission characteristics of 6 ppm of quinine in dioxane-water mixtures. (From Fletcher, ref. 40).


1.0
0.8
0.6
0.4
0.2
0
300
400
500
600
RELATIVE PHOTON EMISSION
WAVELENGTH, NANOMETERS
x3
x3
x30
90% H_2O
50% H_2O
0% H_2O
x10
1% H_2O

glycol as solvent. In the case of tryptophan, the phosphorescence signal was thousand fold smaller for the methanolic solvent compared to the propylene glycol solvent. Whether this result is due to solvent nature or rigidity of the matrix is not clear.

Acidity.--An important characteristic of the environment is the acidity or basicity of the solvent medium. Quinine has been shown by Udenfriend (12) to be relatively non-fluorescent in neutral or basic media. This may be somewhat of an artifact, however. Based on the findings of Fletcher and Chen (loc. cit.) the reduction in intensities in basic media may be due to a spectral shift of the fluorescence peak.

Acidity, nevertheless, is important. Note in Figure 16 that the phosphorescence of nitrophenol is profoundly increased in basic solution as compared to acidic solution. Also, from Figure 14, it is observed that the character (appearance) and position of the peaks of the phosphorescence of quinine is markedly affected by acidity.

Multiple peaks.--Still another feature of the qualitative observations which needs further comment is the appearance of multiple peaks in some of the spectra. This was observed especially in the phosphorescence spectra of quinine (Figure 13), tryptophan (Figure 11) and yohimbine (not shown because tryptophan and yohimbine exhibited almost identical spectra). Although the auxochrome portions of these two molecules differ, the aromatic residue in the two is the same (see Table I). It would seem probable that there must be considerable similarity to the mechanisms leading to and the energies of the preradiational triplet states of the two compounds.

Summary Comments on Environment

The reasons given by various authors (35, 39, 40) for energy shifts of luminescence do not always agree. Chen interprets his findings relating to shifts of emission with shifts in excitation energies on quinine as due to emission from different excited singlet states. Fletcher interprets these same effects as due to rotation of the auxochrome groups. Low temperature blue shifts are believed by Babu and Mugiya to be the result of a frozen in Franck Condon state (actually a viscosity effect). Fletcher and Chen attempt no explanation of the observed blue shift of the emission of quinine with change in solvent.

It is not the purpose of the present study to resolve theoretical controversies. The point to be stressed here is that, depending on the particular situation, there may be one or a number of contributing factors. Because the qualitative observation of the shift in fluorescence of quinine with temperature was observed in this study, it is reported. It is noteworthy that the position (therefore the energy) of the fluorescence of quinine at low temperature and also its intensity is the same whether propylene glycol or methanol is used. This is in contrast to the observation that especially the intensities and in some instances the energies of the phosphorescence peaks depend on the nature of the solvent. Therefore, it would appear that the quantum efficiency of the phosphorescence process is far more dependent on the environmental factors (viscosity and/or temperature) than is the fluorescence process.

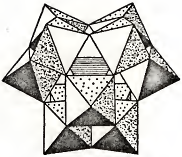
CHAPTER V

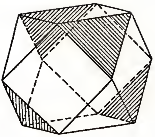
QUALITATIVE LUMINESCENCE STUDIES ON SOLIDS

All of the preceding descriptions relating to the use of FSWC have dealt with studies on liquids, clear, colored, or viscous. One of the original goals of this research was to see if a cell suitable for solids could be used to employ fluorescence and/or phosphorescence for the analysis of drug and alkaloid adducts with the heteropoly anions of phosphorus and silicon. The heteropoly acids (HPA) have been described by Ephraim (41) as "extremely delicate reagents" for the separation of alkaloids and proteins from aqueous solution.

The structure of the heteropoly acids is exceedingly complex, but, due to the exemplary X-rays studies by Keggin (42), a great deal is now known about the structural details. The most common members of this chemical class, the twelve molybdo- and twelve tungsto-silicates and phosphates, have the geometrical form illustrated in Figure 21. The configuration can be considered to be the shape of a cubooctahedron. Triads of molybdic (or tungstic) acid units occupy the triangular faces and these triads are arranged tetrahedrally around the central atom which in the examples under consideration here can be either silicon or phosphorus. The acid forms of these structures exhibit fairly strong acidity, and, probably for this reason and the fact that they have large surfaces, they can accommodate large base molecules as adducts.

Figure 21.--Cubooctahedral configuration of heteropoly anions of silicon and phosphorus (ref. 44).





It has been observed that quenching of fluorescence can occur in the presence of certain heavy atoms, especially the transition metals. For example, Schenk and coworkers (46) have determined Ni(II) by its quenching of the fluorescence of aluminum-1-(2-pyridylazo)-2-naphthol. In another application, Ohnesorge (47) has determined iron at the parts per billion level by the quenching effect on 2,2',2" terpyridine.

A recent finding of interest and one that is pertinent to the consideration here is that of Kirkbright, Narayanaswamy, and West (48). These authors have developed a procedure for the determination of phosphorus by precipitating phosphate in the form of molybdo-phosphoric acid with quinine. The precipitate is redissolved, after washing, in acidified acetone. The fluorescence of quinine after dissolution is measured and related to the phosphorus content of the original mixture.

The fact that quinine remains fluorescent under these circumstances is surprising since in the present study the tungsto- and molybdo-heteropoly complexes of both silicon and phosphorus were found to be quenching agents for quinine in aqueous media. It may be that the complex of quinine and the HPA no longer existsin strong solvents like acetone.

The results of the quenching experiments are given in Table IV.

Quenching is proposed by Hercules (26) and Schenk (46) to be due to an increase of intersystem crossing to the triplet state assisted by the spin-orbit coupling resulting from the presence of the transition metal. If this is the case, then it should be possible to observe phosphorescence of these complex compounds.

TABLE IV

QUENCHING OF QUININE FLUORESCENCE BY HETEROPOLY ACIDS (ROOM TEMPERATURE)

Heteropoly Acid (HPA)	Moles HPA	Moles Quinine	Relative Fluorescence Arbitrary Units
None	---------	1×10^{-7}	72
tungsto-phosphoric	2×10^{-6}	1×10^{-7}	38
tungsto-silicic	2×10^{-6}	1×10^{-7}	7
molybdo-phosphoric	2×10^{-6}	1×10^{-7}	<<1
molybdo-silicic	2×10^{-6}	1×10^{-7}	4

Adducts of two of the analyte molecules, quinine and caffeine, were prepared using 12 tungsto-silicon acid and 12 tungsto-phosphoric acid (Climax Molybdenum Co., New York). The adducts formed readily in acidic solution forming pale yellow fluffy precipitates. On a qualitative basis, it appeared, from a measurement of the relative amounts of the chemicals, that the adducts were generally in the ratio of 3 - 4 molecules of alkaloid to 1 mole of the heteropoly acid (HPA).

The solid adducts were centrifuged and the supernatant liquor drained off. Attempts to wash the separated product resulted in considerable loss of material so washing attempts were abandoned. The products were soft pastes and could be easily scooped from the bottom of the centrifuge tube.

To carry out the luminescence studies, a slightly modified version of the cell design shown in Figure 6 was made. The screen covering for the sample cavity was eliminated and the paste was smoothed into the open well leaving the surface uniformly even with the solid face of the cell. The cell also was used without a positioning pin and was located radially by sight.

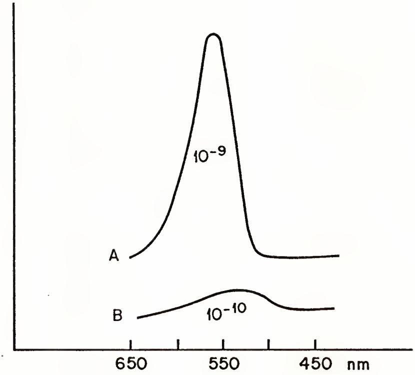
Qualitative studies were carried out both at room temperature and at low temperatures. Broad, ill-defined, low level spectral regions of fluorescence were observed at room temperature with complexes of both quinine and caffeine. The tungsto-phosphate derivatives did not fluoresce, giving signals in most cases only slightly above background. In addition, none of the caffeine adducts showed any noteworthy phosphorescence. Quinine, on the other hand, exhibited an interesting phosphorescence peak as shown in Figure 22. This peak is displaced from its normal position as seen by a comparison with Figure 13.

The results from the luminescent work reported above for the HPA adducts with the alkaloids are not extensive enough to reveal any important applications of these complexes for the purposes under discussion. The studies did however demonstrate the utility of FSWC for the examining the luminescence of solids or pastes.

Figure 22.--Phosphorescence spectrum of quinine adduct with tungsto-silicic acid.

A adduct (paste smoothed into cell cavity)

B blank 1.5 percent solution of heteropoly acid in water


A
10⁻⁹
B
10⁻¹⁰
650
550
450
nm

CHAPTER VI

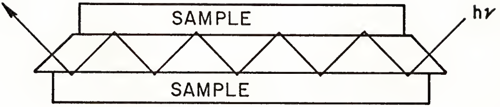
QUALITATIVE RESULTS OF MULTIPLE INTERNAL REFLECTANCE IR STUDIES

Although not directly related to the topics discussed so far, one of the original objectives of this research was to see if infrared analysis could aid in the qualitative analysis of drugs. The thought was that luminescence could serve as the principal quantitative tool and infrared as the qualitative yardstick. Since it would be difficult to separate the analytes in a concentration suitable for infrared analysis from the dilute solutions usually encountered, it was also hoped that the adducts with the heteropoly acids referred to in Chapter V could be used as sequestering agents and the analysis performed on the precipitated solid complexes by multiple internal reflectances.

The use of multiple internal reflectance (MIR) in recent years has been proposed as a way to do qualitative studies on solids (19, 49, 50). The technique of MIR is fairly simple. The infrared analyzing beam is directed into a reflectance plate as shown in the diagram in Figure 23. A sample of the solid is brought into close contact with the surfaces by suitable clamps and the beam is attenuated by this sample contact to give a spectrum which resembles very closely the spectrum which would be obtained by a usual infrared analysis line from solution or a KBr wafer or pellet.

If IR spectra of the adducts turned out to be sensitive and detailed enough to reveal structural aspects of the drugs then, this

Figure 23.--Schematic diagram showing light path through multiple internal reflectance plate. (ref. 50).


SAMPLE
hν
SAMPLE

procedure would serve the qualitative purpose outlined above. Again, caffeine and quinine were picked as representative molecules since they give easily separable adducts. The precipitated products, in this study, were collected on filter paper. The side containing the precipitate was cut into strips and clamped to both sides of a MIR plate. There are a number of choices of plates available as can be seen from Table V.

Because of its wide range of spectral coverage and its ready availability KRS-5 was chosen as the plate to be used for this work.

A comparison of the spectra of the quinine adduct with tungstosilicic acid, filter paper alone and the HPA alone (Sadtler reference Y636K) is shown in Figure 24.

The IR absorption maxima and those obtained by MIR agree closely in position, although in some instances not exactly, with the maxima given for the HPA in the reference spectrum. A small IR absorption shows at about 8.1 μ. An examination of the reference IR absorption spectrum of quinine (Sadtler reference 7546) reveals that a fairly strong absorption peak appears at this point, but there are also even stronger maxima at other positions which are not present in the MIR spectra of the complex. Especially noteworthy is the absence of an absorption maximum at 8.6 μ. It was observed, in comparison, that the corresponding caffeine adduct also exhibited a small absorption peak at 8.1 μ. Therefore, the conclusion is that this is not a very specific nor a very useful peak for qualitative characterization.

That portion of the original goal, then, of using multiple internal reflectance in the infrared as a qualitative aid to the fluorescence study of the drugs considered here was not achieved.

TABLE V

CHARACTERISTICS OF CRYSTALLINE PLATES FOR MULTIPLE INTERNAL REFLECTANCE

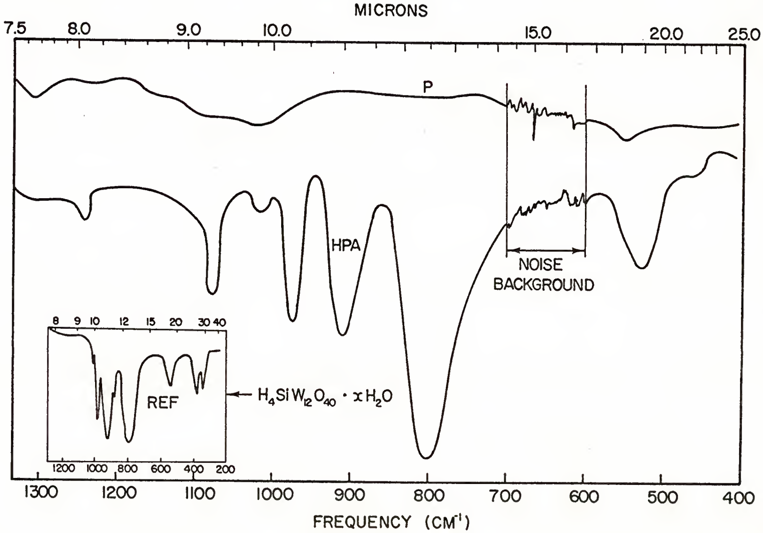
Crystal	Specific Gravity	Refractive Index	Critical Angle	Reflection Losses (2 surfaces) at 10 m.	Useful Wavelength Range μ
Germanium	5.3	4.0	15	52.9	2-11.5
Silicon	2.3	3.5	17	46.1	2- 6.5
TlBr/TlI (KRS-5)	7.3	2.4	24	28.4	1-25
TlBr/TlCl (KRS-6)	7.2	2.2	27	24.2	0.4-25
Silver Chloride	5.5	2.0	30	19.5	1-20

Figure 24.--Comparison of infrared spectra of:

P filter paper, multiple internal reflectance

HPA tungsto-silicic acid adduct with quinine,
multiple internal reflectance

REF tungsto-silicic acid reference spectrum
(Sadtler ref. Y636K, KBr waffer)


MICRONS
7.5
8.0
9.0
10.0
15.0
20.0
25.0
P
HPA
NOISE
BACKGROUND
8
9
10
12
15
20
30
40
REF
1200
1000
800
600
400
200
$H_4SiW_{12}O_{40} \cdot xH_2O$
1300
1200
1100
1000
900
800
700
600
500
400
FREQUENCY (CM^{-1})

CHAPTER VII

PROJECTIONS FOR FUTURE STUDY

As is the experience with the development of any new technique or new device, there have been in this study both successes and some disappointments.

The areas of success include the designing of a useful sample cell arrangement for the study of the luminescence of liquids and solids at room and low temperatures.

Primary among the disappointments was the failure to achieve an operating temperature for the new device lower than -140°C.

Design Modifications

A future design improvement would involve a way to give more flexibility in the choice of operating temperatures.

Part of the lack of success in reaching low temperatures with the present arrangement has been the difficulty of maintaining a constant level of liquid nitrogen in the reservoir. This loss of nitrogen required replacement of the evaporated liquid periodically at intervals of about 15 minutes. To correct this, a liquid level controller should be installed.

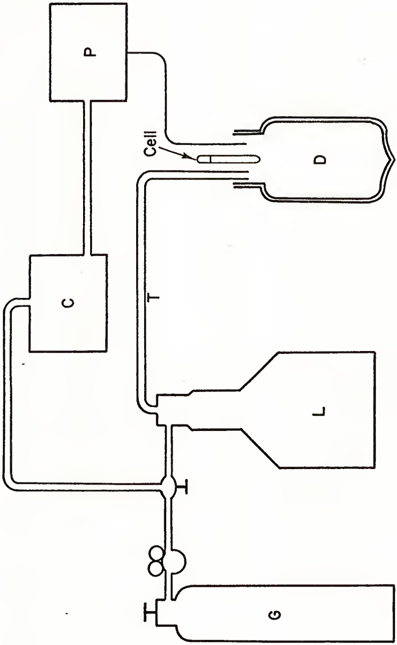
A number of level controllers are available for various cryogenic uses (51, 52, 53, 54). A suitable system, which should serve the purpose desired here and which would use readily available control units, is shown in Figure 25. A modified Dewar flask (for comparison see item D in Figure 7) with a smaller mouth should also be used so that the flask can extend further into the sample chamber. The sensor would be a standard unit (Heraeus - Englehard, reference 51). It is flexible and operates by having two copper sensing elements, one of which dips into the nitrogen and the other just above. Each of the copper elements is independent of the other and provides an electrical contact closure when immersed in the liquid nitrogen.

There is no provision for insulation in the present design. The nitrogen supply tube and the inside of the new housing should be insulated. The insulation in the housing should fill the inside area up to a cylindrical volume of approximately 2 inches in diameter surrounding the cell. The cell should be modified slightly to have a longer base (for comparison see Figure 6). The cell and base, in this modification, would act as an integral cold finger, and a seat consisting of a metal ring anchored to the sides of the housing would position the cell and allow the base to extend below the surface of the liquid nitrogen. A positioning pin provided on the cell circumference would allow radial alignment by semi-cylindrical seats spaced every 5° circularly at five positions (55°, 50°, 45°, 40°, and 35° to the normal of the excitation beam). The cold nitrogen vapors in this setup would have no escape route except upward to surround the limited volume occupied by the sample cell and phosphoroscope.

If desired, temperatures higher than that obtained by having the cell base in direct contact with the liquid nitrogen could be programmed for by moving the operative point of the sensor up or down.

Figure 25.--Schematic arrangement of cell and cooling system with temperature control device.

C	control unit
P	sensor with probe
G	nitrogen gas
L	liquid nitrogen supply
T	transfer line
D	Dewar flask


P
Cell
D
C
T
L
G

A blank cell with a thermocouple sealed in a pool of liquid at the point corresponding to the sample cavity could serve as a calibrating device. The sealed-in liquid could be any mixture showing good heat conductivity.

The results of the above modifications in design would be to 1) bring the sample cavity much closer to the cooling source; 2) provide for better heat transfer; 3) allow for constant replenishment of the nitrogen as it evaporates; 4) add a measure of temperature variability making it possible to study luminescence behavior in relationship to temperature; and 5) provide for optional radial sample positioning.

It should not be necessary with the new design to artificially create an additional volume of nitrogen vapors by bubbling nitrogen gas through the liquid. The extension of the reservoir mouth into the housing and the considerable reduction in housing volume caused by the insulation should mean that the normal vaporization should supply enough vapor volume to exclude moisture.

Although not actually a factor in the design of the cell, an aspect which needs further study is how the spectral reflectance can be reduced. The high backgrounds resulting from reflectance detract from the cell's usefulness at low concentrations. Provision for and installation of polarizers in both exciting and emitting beams along with suitable filters should help to alleviate this problem.

A detailed study should be made of radial positioning. A consequence of the design improvements as noted above is the freedom to rotate the cell to different positions so an evaluation could be made of the optimum setting for different purposes.

Also with the new sample cell assembly provision should be made to allow use of the conventional quartz cell. This would expand the versatility and allow one to do conventional room temperature fluorescence whenever desired. This feature could be easily provided for by installing finger type positioning clamps on the seating ring.

Luminescence Studies

Because there are so many parameters involved in a study of this nature, it was impossible to study all of them or even to investigate thoroughly any one aspect.

It was noted in the course of the discussion that the composition of the solvent had a very marked effect on the magnitude of the signal obtained at low temperatures. It was also observed that at room temperatures highly colored liquids could serve as useful sample media. Considerable further investigation of these aspects needs to be done. Propylene glycol solutions give useful frozen matrices, but the 25 percent solution used in this study may not be optimum. Other polyols could be compared with propylene glycol. Simple freezing experiments revealed, for example, that clear glasses could be obtained with glycol alone (i.e., in the absence of water). Clear glasses have been considered by many of the early investigators (12, 24, 26) to be highly desirable and these glasses should be compared with the snowed media used in this study. Polyethers and viscous hydrocarbons should be evaluated as solvents for analytes not soluble in water.

The use of colored matrices, as mentioned earlier, might serve two purposes. The dark medium would absorb more of the light so that front surface reflection would be less and also the mirror effect of the rear surface of the cell should be entirely eliminated. Application to the direct analysis of whole blood and opaque biological specimens is obvious and will be mentioned in more detail below.

Further Studies on Solids and Viscous Media

The range of applicability of FSWC is almost limitless with regard to the types of samples which can be accommodated for analysis.

An area of growing interest in luminescence as applied to analysis is its use to study biological specimens for the presence of a wide variety of drugs, amino acids, and enzymes (12, 55, 56, 57, 58). If ordinary fluorescence instrumentation is to serve these purposes, the analyte must be separated often by laborious procedures, before analysis.

FSWC offers a way to perform the analytical determinations *in situ*. For example, methods of analytical interest resulting from rates of enzyme reactions as proposed by Guilbault (23, 59) could be performed and monitored directly in the sample cavity of FSWC. The additional advantages would be that special cell pads, such as Guilbault proposes for these analyses, would not be necessary. Also, the study of the influence of temperature on rate of reaction would be facilitated. The substrate could be selected from a wide variety of options, e.g., agar, sugar solutions, and blood plasma.

In the general field of inorganic analysis, it would be of interest to examine the fluorescence behavior of metal chelates. This effort, too, could include such interesting aspects as the effect of varying the structure of the chelate, the solvent temperature, mode of formation, etc.

Crystalline substances could be studied, especially finely divided crystals. For this purpose, the crystals could be made into an agglomerate or thick slurry and smoothed into the sample cavity for luminescence monitoring. Or, if desired, a single crystal could be shaped to fit the sample area and fluorescence or phosphorescence determined.

Quenching can, as has been shown earlier (46, 47), be sometimes used to analytical advantage. Only limited accounts of analytical procedures using quenching are described in the literature. The investigation described earlier in this discourse using the heteropoly acids show that these reagents are quenching agents under certain conditions. Some molecules are quenched more readily than others (26). This fact might be useful in the study of mixtures and the wide applicability of FSWC to solids, e.g., tissue homogenates, would extend the utility of quenching procedures to the field of biological analysis.

Chromatographic procedures are often employed to make separations prior to luminescence analysis. Direct examination of the paper strips or column substrates requires special equipment. FSWC could simplify these procedures. A paper spot containing the desired analyte could be sealed by a drop of liquid to the outside face of the cell and luminescence monitored. Alternatively, powdery substrates from chromatographic columns or sections of thin layer strips could be smoothed into the sample cavity for examination. Spots of interest could easily be examined as sectioned and then treated with various spray reagents either to enhance or selectively diminish (quench) the luminescence. The feature of temperature variability and the ease of changing from fluorescence to phosphorescence merely by a change of housing cover could markedly increase the usefulness of these techniques.

A variation of the above suggestions regarding the study of chromatographic substrates would be to use pellets of various materials. For example, silica, alumina, or carbon particles both active and inactive forms could be formed into pills the size of the sample cavity. Drops of the sample could be introduced onto the surface of the pellet and luminescence studied before and after treatment with eluting agents either directly in the cavity or prior to introduction. These pellets, because they are easily fabricated, would act as inexpensive throw-away sample cells.

The examples cited above of possible ways to use FSWC in useful analytical or qualitative applications is limited only by the imagination of the investigator.

CHAPTER VIII

SUMMARY

Front surface luminescence, although it would seem to have considerable application for spectral analysis, has not been widely used. The principal reason for this is the lack of suitable commercial equipment. Designs for front surface illumination to date have been cumbersome and have restricted usefulness because they apply usually only to specific types of samples, i.e., usually either liquids only or solids only can be accommodated.

The discussion herein has dealt with an entirely new concept of cell design. The range of utility of this new design is broad enough to embrace almost any type of sample (liquid, slurry, paste, or solid) likely to be encountered by the analyst. It also has wide versatility in that it can be used not only for fluorescence studies at room temperature and at reduced temperatures, but, merely by changing the cover on the cell housing, can also be used for phosphorescence studies.

A descriptive account of research illustrating the broad range of applicability of the new cell has been given. Studies at room temperature of fluorescence have shown the cell's usefulness not only for clear, colorless liquids, but also for highly colored ones as well.

Suggestions are given for design modifications to extend the cell's utility to temperatures near that of liquid nitrogen.

Results obtained on qualitative and quantitative studies at temperatures below room temperature (-140°C) include: 1) analytical curves for several drug analytes; 2) qualitative studies to show the application of the cell to the study of the shape and position of the excitation and emission peaks of fluorescence and phosphorescence; and 3) utility for solids and pastes using heteropoly adducts of drug molecules.

Future possible extensions of the above procedures to many areas of analytical interest have been listed to give a sample of avenues for further investigation.

BIBLIOGRAPHY

1. C. A. Parker and W. T. Rees, Analyst, 87, 83 (1962).

2. C. A. Parker, "Photoluminescence of Solutions," Elsevier, New York (1968).

3. American Instrument Co., Inc., Instrument Brochure No. 768F.

4. F. Bertram and W. Kobisch, Chem. Ztg., 91, 809 (1967).

5. H. Jörk, "Quantitative Paper Thin-Layer Chromatography Symposium," E. J. Shellard, ed., Academic Press, London, (1968).

6. B. L. Hamman and M. M. Martin, Anal. Biochem., 15, 305 (1966).

7. T. Hirschfeld, Can. Spectry., 10, 128 (1965).

8. C. A. Parker, Analyst, 94, 161 (1969).

9. E. Kuntz, F. Bishai, and L. Augenstein, Nature, 212, 980 (1966).

10. S. Ainsworth, Anal. Chem., 37, 537 (1965).

11. R. J. Lukasiewicz, P. Rozynes, L. B. Sanders and J. D. Winefordner, Anal. Chem., 44, 237 (1972).

12. S. Udenfriend, Fluorescence Assay in Biology and Medicine, Academic Press, New York, (1962 and Vol II (1969).

13. E. Sawicki and H. Johnson, Microchem. J., 8, 85 (1964).

14. E. Sawicki and J. D. Pfaff, Anal. Chim. Acta., 32, 521 (1965).

15. H. A. Moye and J. D. Winefordner, J. Agr. Food Chem., 13, 533 (1965).

16. T. Hirschfeld, Can. Spectry., 11, 102 (1966).

17. Ibid., 11, 115 (1966).

18. T. Hirschfeld, Appl. Optics, 6, 715 (1967).

19. J. Fahrenfort, Spectrochim. Acta, 17, 698 (1961).

20. S. Ainsworth and E. Winter, Appl. Opt., 3, 371 (1964).

21. G. G. Guilbault and A. Vaughan, Anal. Chim. Acta., 55, 107 (1971).

22. G. G. Guilbault and R. Zimmerman, Anal. Lett., 3, 133 (1970).

23. G. G. Guilbault and A. Vaughan, Anal. Lett., 3, 1 (1970).

24. G. G. Guilbault, "Fluorescence - Theory, Instrumentation and Practice," Marcel Dekker, New York (1967).

25. A. J. Pesce, C. G. Rosén, T. L. Pasby, "Fluorescence Spectroscopy," Marcel Dekker, New York (1971).

26. D. M. Hercules, "Fluorescence and Phosphorescence Analysis," Interscience (1966).

27. J. D. Winefordner, P. S. St. John, and W. J. McCarthy, "Phosphorimetry as a Means of Chemical Analysis," Vol. II, Fluorescence Assay in Biology and Medicine, S. Udenfriend, Ed., Academic Press, New York (1969).

28. R. F. Chen, Anal. Biochem., 14, 497 (1966).

29. W. H. Melhuish, J. Phys. Chem., 65, 229 (1961).

30. R. Rusakowicz and A. C. Testa, J. Phys. Chem., 72, 793 (1968).

31. R. P. Simons, "Photochemistry and Spectroscopy," Wiley-Interscience, London; New York (1971).

32. R. Zweidwinger and J. D. Winefordner, Anal. Chem., 42, 639 (1970).

33. F. C. Lim, J. D. La Posa and J. M. H. Yu, J. Mol. Spectry., 19, 412 (1966).

34. E. J. Bowen and J. Sahu, J. Phys. Chem., 63, 4 (1959).

35. H. Babu and C. Mugiya, J. Chem. Soc. of Japan, Bull., 43, 13 (1970).

36. American Instrument Co., Inc., Instrument Brochure No. 816A.

37. E. J. Bowen, Trans. Faraday Soc., 50, 97 (1954).

38. E. L. Wehry, Structural and Environmental Factors in Fluorescence, Chap. 2, "Fluorescence--Theory, Instrumentation and Practice," G. G. Guilbault, Ed., Marcel Dekker, New York (1967).

39. R. F. Chen, Anal. Biochem., 19, 374 (1967).

40. A. N. Fletcher, J. Phys. Chem., 72, 2742 (1968).

41. F. Ephraim, "Inorganic Chem.," P. C. L. Thorne and E. R. Roberts, ed., Interscience, New York (1943).

42. J. F. Keggin, Nature, 131, 908 (1933); Proc. Roy. Soc. (London), A 144, 75 (1934).

43. A. F. Wells, "Structural Inorganic Chemistry," 3rd ed., The Clarendon Press, Oxford (1962).

44. G. A. Tsigdinos, "Heteropoly Compounds of Molybdenum and Tungsten," Bull Cdb-12a, Climax Molybdenum Co., New York (1966).

45. J. W. Linnett, J. Chem. Soc., 1961, 3796.

46. G. H. Schenk, K. P. Dilloway and J. S. Coulter, Anal. Chem., 41, 510 (1969).

47. D. W. Fink, J. V. Pivnichny and N. E. Ohnesorge, Anal. Chem., 41, 833 (1969).

48. G. F. Kirkbright, R.Narayanaswamy, and T. S. West, Analyst, 97, 174 (1972).

49. R. O. Crisler, Annual Reviews, Anal. Chem., 42, 388R (1970).

50. Bulletin entitled "Internal Reflection Spectroscopy," Vol. 1, Wilks Scientific Company, South Norwalk, Conn. (1965).

51. Heraeus - Englehard, Vacuum, Inc., Bulletin 1101.

52. Advanced Products Dept., Air Products and Chemicals, Inc., Cat. No. CRS - 571/4M.

53. Matheson Gas Products, Bulletin G-814/UL/10M/371.

54. Linde Air Products, Bulletin F3239.

55. C. E. White and A. Weissler, Annual Reviews, Anal. Chem., 44, 182R (1972).

56. K. Samejima, W. Dairman and S. Udenfriend, Anal. Biochem., 42, 222 (1971).

57. Ibid., 42, 237 (1971).

58. S. J. Mule and P. L. Hushin, Anal. Chem., 43, 708 (1971).

59. G. G. Guilbault, "Enzymatic Methods of Analysis," Pergamon Press, New York (1920).

60. S. Sharafy and K. A. Muszkat, J. Amer. Chem. Soc., 93, 4119 (1971).

61. J. D. Winefordner and M. Tin, Anal. Chim. Acta., 32, 64 (1965).

62. B. Meyer, "Low Temperature Spectroscopy," Elsevier, New York (1971).

BIOGRAPHICAL SKETCH

James Alvah McHard was born in Aledo, Illinois on October 25, 1914. He attended high school there and obtained his B.S. degree in 1935, and his M.S. degree in 1936 from the University of Illinois. He entered on an industrial career with Dow Chemical Company, Midland, Michigan, as a chemist in November 1936. When Dow Corning Corporation was formed he transferred to that company as Analytical Group Leader and later as Manager of the Analytical Services Department. He continued in that capacity until 1969 when he left to enter the University of Florida to complete his academic work.

He has participated in a number of civic organizations and projects during the last thirty-five years. He is a member of the American Chemical Society and the Division of Analytical Chemistry.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James D. Winefordner

James D. Winefordner, Chairman
Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

L. A. Arnold

Luther A. Arnold
Associate Professor of Education

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Roger G. Bates

Roger G. Bates
Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Gerhard M Schmid

Gerhard M. Schmid
Associate Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

R Carl Stoufer

R. Carl Stoufer
Associate Professor of Chemistry

This dissertation was submitted to the Department of Chemistry in the College of Arts and Sciences and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1972

Dean, Graduate School